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Proteolytic action of kallikrein-related peptidase 7 produces unique active matrix metalloproteinase-9 lacking the C-terminal hemopexin domains

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ABSTRACT

The gelatinases, matrix metalloproteinase (MMP)-9 and -2, are produced as latent, inactive enzymes that can be proteolytically activated by a number of proteases. In many normal and pathological conditions, where the expression of MMPs is deregulated, changes in the expression of other proteases have also been reported. Human kallikrein-related peptidase 7 (KLK7), a chymotryptic-like serine protease, is overexpressed in many different types of neoplastic conditions, which have also been shown to express high levels of both MMP-9 and -2. Since the activation of MMPs by KLK7 has never been examined, we sought to determine whether KLK7 can activate these MMPs. To test this hypothesis KLK7 was incubated with the recombinant MMPs and the products of the reaction were analyzed for their activity. Incubation of proMMP-9 with KLK7 resulted in the production of a novel truncated, active MMP-9 lacking the C-terminal hemopexin domains. In contrast, KLK7 degraded, but did not activate, proMMP-2. The novel activation of proMMP-9 by KLK7 was further confirmed using conditioned medium prepared from an MMP-9-expressing cell line, MDA-MMP-9. Our results clearly establish that KLK7 activates proMMP-9 to produce a novel truncated, active MMP-9 product not generated by other proteases. These findings suggest that KLK7 may play an important role in the activation of MMP-9 in tumors that express high levels of both these proteases and the resulting truncated MMP may possess altered substrate specificities compared with full-length MMP-9 activated by other proteases.

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1. Introduction

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent, neutral endopeptidases that participate in the degradation of the extracellular matrix and remodeling in both normal and pathological conditions, including cancer. During metastasis, tumor cells must detach from the primary tumor, migrate, and invade surrounding tissues—processes that are actively facilitated by MMPs. Members of the MMP family have been studied in detail for their ability to influence the progression of different types of cancers [1–3]; and various MMPs have been shown to regulate tumor progression by proteolysis of extracellular matrix proteins [4], remodeling the tumor microenvironment [5], processing cell–cell adhesion molecules [6], selecting tumor cells resistant to apoptosis [7], and promoting tumor angiogenesis [8]. In particular, degradation of the basement membrane, comprised predominately of type IV collagen, is carried out by the gelatinases (MMP-2 and MMP-9).

MMPs are secreted as latent, inactive proenzymes that can be converted into active enzymes by proteolytic removal of the propeptide domain containing a highly conserved cysteine residue that forms an intramolecular complex with the active site zinc atom [9,10]. Many extracellular proteases have been demonstrated to directly activate MMPs including other MMPs (e.g., MMP-3 activation of the MMP-9 [11]) and non-MMP proteins (e.g., plasmin activation of MMP-3 [12]). High levels of metalloproteinases, especially MMP-2 and/or MMP-9 have been demonstrated in many cancers, including pancreatic [13–20], breast [21], cervical [22], and ovarian cancer [23]. Similarly, the human kallikrein-related peptidases constitute an important group of serine proteases that are up-regulated in various cancers (reviewed in [24,25]). In particular, kallikrein-related peptidase 7 (*KLK7*) has also been reported to be overexpressed in human pancreatic [26], breast [27], cervical [28], and ovarian cancer [29–31]. Dysregulated KLK expression in neoplastic tissues may play a role in tumor growth, invasion, metastasis, and angiogenesis (reviewed in [24,32,33]). The co-expression of *KLK7* and *MMP*-2 and -9 prompted

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us to investigate whether KLK7 could activate these MMPs. Results from our experiments clearly show that KLK7 can activate purified proMMP-9, but not proMMP-2, *in vitro* to produce a novel active MMP-9 gelatinolytic fragment that lacks the C-terminal hemopexin domains, which is in contrast to the 83-kDa product produced by other proteases. Kallikrein-related peptidase 7 was also able to activate proMMP-9 secreted in conditioned medium by MDA-MMP-9 cells in a similar fashion. These results highlight a novel role for KLK7 in the activation of MMP-9 in tissues where both these proteases are highly expressed and should temper the assignment of gelatinolytic activity based solely on gel mobility in mixtures of MMPs. Importantly such activation of MMP-9 by KLK7 in pathological conditions, like cancer, could result in truncated MMP-9 that may exhibit altered substrate specificity and not be targeted by current therapies.

2. Materials and methods

2.1. Cell culture and conditioned media

MDA-MMP-9 cells (kindly provided by Dr. James P. Quigley) and the parental breast cancer cell line MDA-MB-231 were seeded in 10-cm dishes and grown to 70% confluence in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) at 37 °C in a 5% CO_2/air environment. Geneticin (600 μg/ml) was included in the culture medium for the MDA-MMP-9 cells. For preparation of conditioned media, growth media was removed, cells were washed twice in phosphate-buffered saline, and incubated in serum-free medium (SFM) for 48 h. After incubation, the conditioned media was removed, centrifuged at 1500 rpm at 4 °C to remove any cell debris, aliquoted, and stored at −20 °C.

2.2. Activation of proKLK7

Recombinant, proKLK7 (100 μg/mL) (R&D Systems, Minneapolis, MN) was proteolytically activated using thermolysin as described previously [34]. To activate with plasmin, equal molar amounts of proKLK7 and plasmin (EMD Chemicals) were incubated at 37 °C for 4 h in 50 mM Tris–HCl, pH 7.2, 0.15 M NaCl. Plasmin activity was terminated by addition of D-Val-Phe-Lys chloromethyl ketone (VFK-CK, EMD Chemicals), a selective irreversible plasmin inhibitor.

2.3. Gelatin zymography

For gelatin zymography, mixtures after incubation were mixed with non-reducing SDS-PAGE sample buffer and incubated at RT for 10 min. The samples were then resolved in 15% or 20% acrylamide SDS-PAGE gels containing 10 mg/ml of porcine gelatin. After electrophoresis, the gels were washed for 1 h in renaturing buffer (2.5% Triton X-100) and incubated overnight in developing buffer (50 mM Tris, pH 7.5, 200 mM NaCl, and 5 mM $CaCl_2$) at 37 °C with constant shaking. The gels were then stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA) in 50% methanol and 10% acetic acid overnight. The bands were visualized after repeated washes with a 50% methanol, 10% acetic acid solution.

2.4. Activation of proMMP-9 and proMMP-2 by KLK7

ProMMP-9 and proMMP-2 (1 pmol) (EMD Biosciences, La Jolla, CA) were incubated with 0.2 pmol of thermolysin-activated KLK7 at 37 °C in KLK7 activity buffer (50 mM Tris–HCl, pH 8.5, 0.15 M NaCl) containing 50 mM EDTA (to inhibit thermolysin). At various time intervals samples were removed and resolved on 4–12% Bis-Tris polyacrylamide gels (Invitrogen) for western blots or 20% polyacrylamide gels containing 0.1% gelatin to monitor gelatinolytic activity. As controls, proMMP-9 or proMMP-2 alone or with 1 ng of thermolysin, were incubated under similar conditions separately for 4 h. As a further control, thermolysin-activated KLK7 was incubated in the presence of 50 mM EDTA for 4 h.

2.5. Activation of proMMP-9 by KLK7 in conditioned medium of MDA-MMP-9 cells

Conditioned media (20 μl) from MDA-MMP-9 cells was incubated with 100 ng of thermolysin-activated KLK7 in the presence of 50 mM EDTA at 37 °C for 0, 30, 60, 120, 240 minutes. At each time point, an aliquot was removed for gelatin zymography. As controls, 20 μl of MDA-MMP-9 conditioned media was incubated alone or with 10 ng of thermolysin in presence of 50 mM EDTA at 37 °C for 4 h. As a positive control, 1 pmol of recombinant proMMP-9 was incubated with 0.2 pmol of thermolysin-activated KLK7 in 20 μl of SFM containing 50 mM EDTA for 4 h at 37 °C.

2.6. Activation of recombinant proMMP-9 by KLK7 in MDA-MB-231 conditioned media

ProMMP-9 (1 pmol) was incubated with 0.2 pmol of thermolysin-activated KLK7 in 20 μl of MDA-MB-231 conditioned media in the presence of 50 mM EDTA at 37 °C. As controls, 20 μl of conditioned media was incubated alone, with 100 ng proMMP-9, or with 100 ng proMMP-9 and 1 ng thermolysin at 37 °C in the presence of 50 mM EDTA. As a positive control, proMMP-9 was incubated with thermolysin-activated KLK7 in 20 μl of SFM containing 50 mM EDTA at 37 °C. After 4 h, the samples were analyzed by gelatin zymography, visualized by Coomassie Blue staining, and the sizes of the novel gelatinolytic fragments were determined using an AlphaEase image documentation system and analysis software (Alpha Innotech, San Leandro, CA).

2.7. Activation of proMMP-9 by KLK7 in TIMP-1-immunodepleted conditioned medium of MDA-MB-231 cells

MDA-MB-231 conditioned medium (500 μl) was incubated with 2 μg of anti-TIMP-1 antibody (R&D Systems) on a rotating mixer overnight at 4 °C. As a control, 500 μl of conditioned medium was incubated with 2 μg of normal goat IgG antibody (R&D Systems) under similar conditions. Immunocomplexes were removed by addition of a 50% slurry of protein G Sepharose 4 Fast Flow (GE Healthcare, Piscataway, NJ), mixing for 2 h at 4 °C, followed by centrifugation. ProMMP-9 (1 pmol) was incubated with 0.2 pmol thermolysin-activated KLK7 in 20 μl of TIMP-1-immunodepleted MDA-MB-231 conditioned medium or control IgG-treated conditioned medium in the presence of 50 mM EDTA for 4 h at 37 °C. As controls, 20 μl of TIMP-1 or control immunodepleted conditioned medium was incubated alone, with 1 pmol of proMMP-9, or with 1 pmol of proMMP-9 and 1 ng thermolysin in presence of 50 mM EDTA for 4 h at 37 °C. As a positive control, 1 pmol of proMMP-9 was incubated with 0.2 pmol thermolysin-activated KLK7 in 20 μl of SFM containing 50 mM EDTA for 4 h at 37 °C. At the end of incubation, one aliquot was subjected to gelatin zymography and a second aliquot was analyzed by western blot for the presence of TIMP-1.

2.8. Western blot analysis of KLK7-cleaved proMMP-9

For detection of proMMP-9 products cleaved by KLK7 using western blot analysis, PVDF membranes were incubated with MMP-9 antibodies generated using full-length proMMP-9 (AF911, R&D Systems), the catalytic domain of MMP-9 (AB19016, Millipore), or peptide immunogens located in the C-terminus of MMP-9 (M5177, Sigma-Aldrich; 56-2A4, Millipore).

2.9. N-terminal sequence analysis of KLK7-cleaved MMP-9

To determine the KLK7 cleavage site of MMP-9, 50 pmol of proMMP-9 was incubated with 10 pmol of thermolysin-activated KLK7 for 4 h at 37 °C. The reaction was terminated by heating at 95 °C for 5 minutes following the addition of SDS-PAGE sample buffer. The products were resolved on a 14% acrylamide/SDS gel, transferred to PVDF membrane, and visualized with 0.2% Ponceau-S stain in 1% acetic acid. The membrane with truncated ~48-kDa band was excised, washed in methanol followed by distilled water, and submitted to the Harvard Microchemistry Facility for protein sequence analysis.

3. Results

Since *MMPs*-2 and -9 and *KLK7* have been observed to be overexpressed in various cancers, we examined the ability of KLK7 to activate these MMPs. Upon incubation, recombinant proMMP-9 was cleaved by KLK7 in a time-dependent manner giving rise to a distinct truncated, proteolytically active product as visualized by gelatin zymography (Fig. 1A). By 4 h, the full-length 92-kDa proMMP-9 was almost entirely cleaved by KLK7 resulting in a low molecular weight gelatinase activity (Fig. 1A, arrowhead labeled *tMMP-9*). To confirm that the gelatinolytic activity resulted from MMP-9 activated by KLK7 and not by the thermolysin used to activate KLK7, proMMP-9 was incubated without and with thermolysin in the presence of EDTA (Fig. 1A, lanes 7 and 8, respectively). Although thermolysin exhibited significant gelatinase activity (Fig. 1A, arrow labeled *therm*), it did not produce the truncated, active MMP-9 band observed in the presence of KLK7. Similarly, gelatin zymography of thermolysin-activated KLK7 only elicited gelatinolytic activity from the thermolysin and not KLK7 (Fig. 1A, lane 9). To inhibit thermolysin activity, EDTA was included in the reactions during the incubation period; however, thermolysin activity was restored following electrophoresis as indicated by its robust gelatinolytic activity in the control reactions. To rule out the possibility that EDTA in the reactions could have influenced the conformation of MMP-9 leading to this unique activation with KLK7, we tested MMP-9 activation using plasmin-activated KLK7. Gelatin zymography of the reaction products revealed that a similar truncated, gelatinolytically-active MMP-9 fragment was produced with either plasmin-activated KLK7 or thermolysin-activated KLK7 (data not shown); thus, eliminating the notion that EDTA influences the activation of proMMP-9 by KLK7. In contrast to the activation of MMP-9 by KLK7, incubation of proMMP-2 with KLK7 resulted in a time-dependent degradation of MMP-2 gelatinase activity without yielding any distinct major gelatinolytic fragments (Fig. 1B). Thus, among these MMPs, activation by KLK7 appears to be specific for MMP-9.

To determine the site of cleavage by KLK7 to produce the truncated MMP-9, proMMP-9 was incubated with KLK7 and the reaction products were loaded in two lanes of a 14% SDS-PAGE gel. Following electrophoresis, the two lanes were divided and one was stained with Coomassie Blue to visualize the reaction products (Fig. 2) while the other was transferred to PVDF membrane and stained with Ponceau S. The major band observed, corresponding to the truncated MMP-9 band detected by Coomassie Blue (Fig. 2, arrowhead labeled *tMMP-9*), was excised and submitted for N-terminal sequence analysis. Five cycles of Edman degradation performed on the KLK7-cleaved MMP-9 yielded the sequence(s) T/[G]-P-R-P-E (amino acid in brackets reported with reasonable confidence). The N-terminal sequence G-P-R-P-E was verified in a second preparation, however, no sequence data were obtained for the 51-kDa catalytic domain in either preparation, presumably because its N-terminus is blocked. Inspection of the proMMP-9

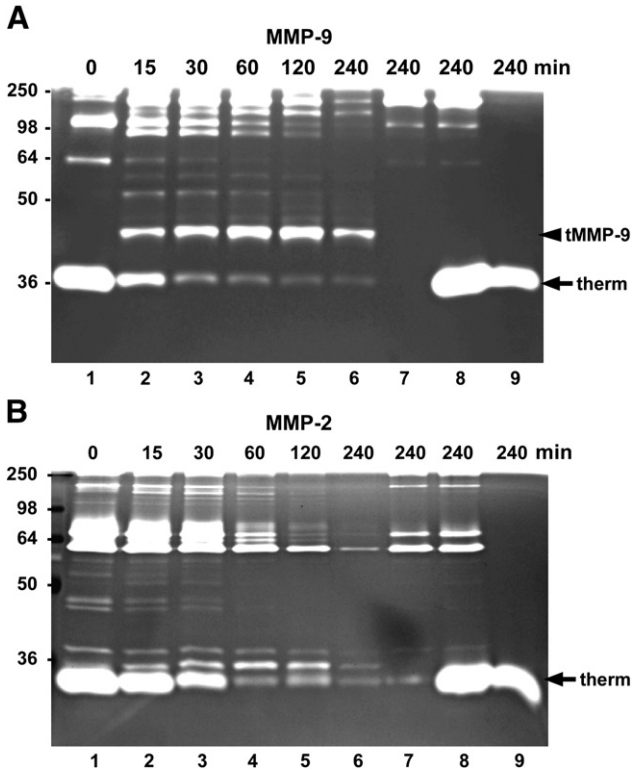

Fig. 1. ProMMP-9 but not proMMP-2 is activated by KLK7. (A) Recombinant proMMP-9 (1 pmol) or (B) proMMP-2 was incubated with 0.2 pmol of thermolysin-activated KLK7 at 37 °C for the indicated times (lanes 1–6) in activity buffer containing 50 mM EDTA and the products were separated by SDS-PAGE and visualized by gelatin zymography. As controls, 1 pmol of the corresponding proMMP was incubated without (lane 7) or with thermolysin (lane 8) in the presence of EDTA (to inhibit thermolysin activity) for 4 h, or 0.2 pmol of thermolysin-activated KLK7 was incubated for 4 h (lane 9) prior to gel analysis. The predominant proteolytically active truncated MMP-9 (tMMP-9) fragment produced by KLK7 is denoted with arrowheads and gelatinolytic activity corresponding to thermolysin (therm) is indicated by the arrows. Sizes of the protein markers (kDa) are indicated on the left.

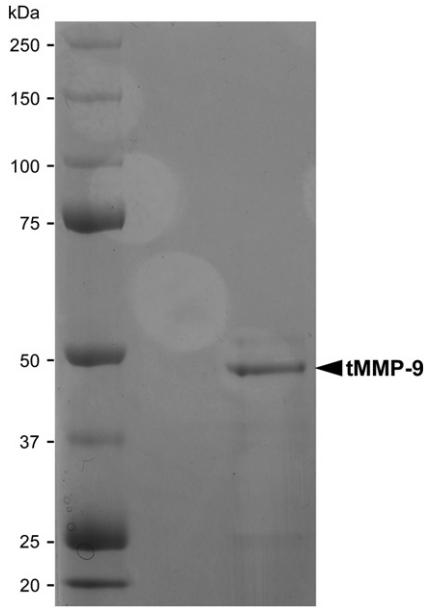

Fig. 2. N-terminal sequence analysis. Recombinant proMMP-9 was incubated with activated KLK7 and the reaction products were fractionated on a 14% Tris-glycine gel. Following electrophoresis the gel was divided and the reaction products in one portion were visualized by Coomassie Blue stain. Sizes of the protein markers (kDa) are indicated on the left. The other gel portion was transferred to PVDF membrane, stained with Ponceau S and the membrane containing the major truncated MMP-9 (tMMP-9) product (corresponding to the band visualized by Coomassie Blue) was excised and submitted for N-terminal sequence analysis yielding the sequence GPRPE.

sequence revealed the sequence G-P-R-P-E (residues 444–448, Fig. 3A) located between the consensus HEFGHALGLDH catalytic domain motif and hemopexin-like domains (HP) in the C-terminus. This sequence is preceded by a tyrosine residue, the most favored P1 residue of KLK7 as determined by substrate specificity profiling [35], supporting the notion that this represents a KLK7 cleavage site.

It is also noteworthy that catalytically active recombinant forms of MMP-9 have been produced that were truncated following the protease domain at almost the same site (GPRPE) cleaved by KLK7 [36,37]. Intriguingly, the mobility of the catalytically active portion of MMP-9 produced by KLK7 identified by gelatin zymography is very similar to that observed for truncated murine MMP-9 [36].

To further characterize the MMP-9 cleavage products produced by KLK7, proMMP-9 was incubated with KLK7 and the reaction products were loaded in multiple lanes of 4–12% Bis-Tris gels. Following transfer to PVDF membranes the cleavage of proMMP-9 by KLK7 was assessed by western blot analysis using various MMP-9 antibodies (Fig. 3B). Using an antibody (AF911, R&D Systems) generated against recombinant proMMP-9, two proteolytic products were detected corresponding to 51 and 48 kDa. Immunoblots incubated with an antibody targeting the catalytic domain of MMP-9 (AB19016, Millipore) revealed that the 51-kDa band represents the novel truncated MMP-9 fragment observed by gelatin zymography (Fig. 1A, arrowhead labeled *tMMP-9*). Consistent with this finding, antibodies produced using peptide immunogens derived from the C-terminus of MMP-9 detected the 48-kDa band, which represents the C-terminal portion of MMP-9 containing the hemopexin domains. This C-terminal fragment would be produced by cleavage between Tyr443 and Gly444 yielding the N-terminal sequence GPRPE that was identified by Edman sequencing. Due to their similar size, the 51- and 48-kDa MMP-9 fragments were only resolved using 4–12% gradient Bis-Tris gels (Fig. 3B) and not when the reaction products were fractionated using a 14% Tris-glycine gel (Fig. 2).

To determine whether KLK7 can activate proMMP-9 in the context of other secreted cellular products, conditioned medium was prepared from MDA-MMP-9 cells, which are MDA-MB-231 cells, derived from a breast carcinoma, transfected to express high levels of MMP-9 [11], but do not express KLK7 [38]. Upon incubation with KLK7, proMMP-9 in the conditioned medium was efficiently cleaved by KLK7 to yield a 51-kDa truncated gelatinolytic fragment (Fig. 4, arrowhead), which is similar to that observed with purified, recombinant MMP-9 (Fig. 1A). Interestingly, an additional gelatinolytically-active MMP-9 band was also seen in experiments using the MDA-MMP-9 conditioned medium. Both fragments were also produced in a control reaction when recombinant proMMP-9 was incubated with KLK7 in SFM rather than KLK7 activity buffer (Fig. 4, lane 8, arrowheads), suggesting that an additional site within MMP-9 becomes susceptible to cleavage by KLK7 in these buffer conditions. Neither the conditioned medium alone nor conditioned medium with thermolysin produced these active MMP-9 products (Fig. 4, lanes 6 and 7, respectively).

To identify soluble factors in conditioned media that may regulate KLK7-mediated activation of MMP-9, MDA-MB-231 cells were employed for further *in vitro* activation studies. MDA-MD-231 cells, the parental cells transfected to produce high levels of MMP-9, did not exhibit detectable levels of MMP-9 as measured by gelatin zymography (Fig. 5A, lane 1). These cells should possess the same cellular factors regulating activation of MMP-9 as the MDA-MMP-9 cells and thus provide a context for manipulating the exogenous levels of both KLK7 and MMP-9. Interestingly, in the presence of MDA-MB-231 conditioned medium KLK7 did not activate recombinant proMMP-9 in a fashion similar to that observed with SFM (Fig. 5A, compare lanes 4 and 5). Incubation with active KLK7 produced only low levels of a ~51-kDa fragment (Fig. 5A, lane 4). These results indicate that there is a factor in the conditioned medium that regulates KLK7 activation of proMMP-9.

One of the secreted factors that has been studied extensively in regulating the activation, as well as activity, of MMPs is TIMP-1 (tissue inhibitor of metalloproteinase-1) [39]. To test the role of TIMP-1 in KLK7-mediated activation of proMMP-9, proMMP-9 was incubated with KLK7 in MDA-MB-231 conditioned medium with (Fig. 5B) and without (Fig. 5C) immunodepletion of TIMP-1. Immunodepletion of TIMP-1 from MDA-MB-231 conditioned medium resulted in the formation of both truncated MMP-9 products (Fig. 5B, lane 4, arrowheads), similar to the gelatinolytic bands produced with SFM (Fig. 5B, lane 5). Parallel reactions performed with MDA-MB-231 conditioned medium immunodepleted with normal goat IgG exhibited almost complete attenuation of

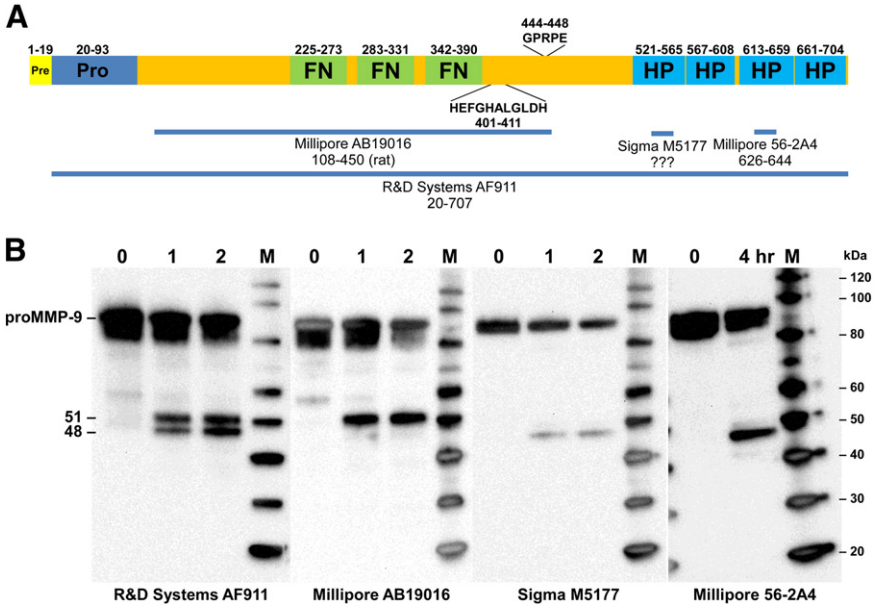

Fig. 3. Western blot analysis of proMMP-9 cleavage products. (A) Schematic diagram of preproMMP-9 depicting the locations of the fibronectin (FN), zinc-binding catalytic (HEFGHALGLDH), and hemopexin (HP) domains. The MMP-9 immunogens used to generate the MMP-9 antibodies used for western analysis are indicated by the horizontal lines (B) Recombinant proMMP-9 was incubated with activated KLK7 for the indicated times and the reaction products were loaded in multiple lanes of 4–12% Bis-Tris gels. Following electrophoresis, the products were transferred to PVDF membranes and detected with the indicated MMP-9 antibodies. MagicMarkXP (Invitrogen) protein standards (M) were used to determine the size of the MMP-9 products. Sizes of the protein markers (kDa) are indicated on the right.

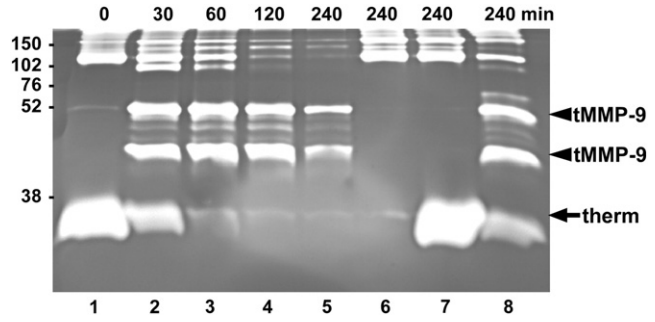

Fig. 4. KLK7 activates proMMP-9 secreted by MDA-MMP-9 cells. Conditioned medium from MDA-MMP-9 cells was incubated at 37 °C with 100 ng of thermolysin-activated KLK7 in the presence of 50 mM EDTA for the indicated times (lanes 1–5). As controls, conditioned medium was incubated without (lane 6) or with thermolysin (lane 7) under similar conditions for 4 h. As a positive control, 1 pmol of proMMP-9 was incubated with 0.2 pmol thermolysin-activated KLK7 (lane 8) in SFM containing EDTA for 4 hrs. At the end of the incubation period the products were analyzed by gelatin zymography. The truncated MMP-9 (tMMP-9) gelatinolytic products produced by KLK7 are denoted by the arrowheads and thermolysin-directed proteolysis (therm) is indicated by the arrow. Sizes of the protein markers (kDa) are indicated on the left.

MMP-9 activation by KLK7 (compare Fig. 5B, lane 4 and Fig. 5C, lane 4). The removal of TIMP-1 from the conditioned medium (Fig. 5B, lower panel) and its retention with control immunoglobulins (Fig. 5C, lower panel) was verified by western blot. These results clearly show that TIMP-1 can regulate KLK7-mediated cleavage of MMP-9.

4. Discussion

Matrix metalloproteinases play a key role in the progression of many disease conditions including cancer. The members of this family—including the collagenases, stromelysins, gelatinases, and membrane-type metalloproteases—are zinc-containing, neutral endopeptidases, comprised of multiple domains that are characterized by their ability to degrade various extracellular proteins. Both the expression and the activity of MMPs are regulated and controlled at different levels [40]. Matrix metalloproteinases, like MMP-9, have been found to be expressed by various normal cell types and also by certain cancer cells [41–43]. Previous studies have shown that MMP-9 is a key player in physiological processes in both normal and disease conditions. Higher MMP-9 expression has also been observed in many different types of cancer [21–23] in which both microenvironment- [44,45] and tumor-derived MMP-9 [46] have been shown to influence tumor cell invasion, metastasis [47,48], intravasation [49], and angiogenesis [1,50].

One of the key mechanisms leading to activation of proteases, in both normal and diseased conditions, is via proteolytic cascades involving the activation of one protease leading to further activation of proteases from same and other protease families. Such proteolytic activation cascades are known to play an important role in processes such as extracellular matrix degradation, which leads to tumor invasion [51] and progression of many different neoplastic conditions [52]. Most of the MMPs are secreted as latent zymogens and are activated by the proteolytic removal of the propeptide domain [10]. Previous studies have shown that many proteases, such as trypsin [53], plasmin [54,55], and chymase [56] as well as other MMPs (e.g., MMP-3 [57], MMP-7 [58]), can activate proMMP-9 *in vitro*.

KLK7, a chymotryptic-like serine protease, was initially purified and characterized from human skin extracts and is thought to play a role in desquamation of human skin [59,60], however, increasingly the *KLK7* transcript and/or KLK7 protein has been found to be overexpressed in human cancers. Previously, we reported that KLK7 is overexpressed in pancreatic adenocarcinomas [26]. Other laboratories have also demonstrated that it is overexpressed in ovarian [29–31,61], squamous cervical [28], and breast cancers [27]. Interestingly, in many of these cancers there is a parallel in the overexpression of KLK7 and MMPs, such as MMP-9 and MMP-2 [62–64]. Based on these observations, we sought to determine whether KLK7 could activate proMMP-9 and proMMP-2.

Using a series of *in vitro* experiments we found that KLK7 was able to cleave proMMP-9 to produce a novel truncated fragment with gelatinolytic activity in a time-dependent manner. Activation of the 92-kDa proMMP-9 by other proteases typically yields an 83-kDa active protease. Using a fivefold molar excess of substrate (proMMP-9) to enzyme (KLK7), KLK7-cleaved MMP-9 products exhibited robust gelatinolytic activity that was clearly evident within 15 minutes and the proMMP-9 was completely activated by 4 h. This rapid activation of proMMP-9 by KLK7 is reminiscent of the activation reported for MMP-3 using similar reaction conditions [57] and appears much more efficient than activation by trypsin [53]. Furthermore, MMP-3 has been demonstrated to generate an active 82-kDa form of MMP-9 that is subsequently converted into a major gelatinase with a mass of 35 kDa

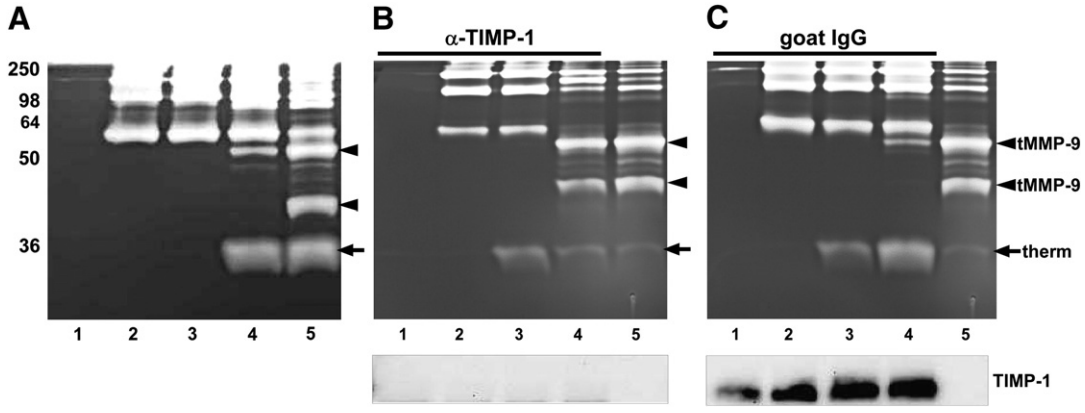

Fig. 5. TIMP-1 in MDA-MB-231 conditioned medium regulates KLK7 activation of proMMP-9. (A) MDA-MB-231 conditioned medium containing 50 mM EDTA was incubated at 37 °C for 4 h with no additions (lane 1), with 1 pmol proMMP-9 (lane 2), with 1 pmol proMMP-9 and thermolysin (lane 3), or with 1 pmol proMMP-9 and 0.2 pmol thermolysin-activated KLK7 (lane 4). As a positive control, 1 pmol proMMP-9 was incubated with 0.2 pmol thermolysin-activated KLK7 in SFM containing EDTA at 37 °C for 4 h (lane 5). At the end of the incubation period, the samples were analyzed by gelatin zymography. MDA-MB-231 conditioned medium was immunodepleted with a TIMP-1 antibody (B) or control goat IgG (C). The immunodepleted conditioned medium was incubated with the additions indicated in (A) at 37 °C for 4 h prior to analysis by gelatin zymography (upper panels) or western blot for TIMP-1 (lower panels). As a positive control, 1 pmol proMMP-9 was incubated with 0.2 pmol thermolysin-activated KLK7 in SFM containing EDTA at 37 °C for 4 h (lane 5). The truncated MMP-9 (tMMP-9) gelatinolytic products are denoted by the arrowheads and thermolysin-directed proteolysis (therm) is indicated by the arrow. Sizes of the protein markers (kDa) are indicated on the left.

[65]. Intriguingly, in a study using a transgenic mouse model of pancreatic islet carcinogenesis, an unidentified 43-kDa gelatinolytic activity was observed during the angiogenic switch triggered by MMP-9 [66], similar in size to the novel gelatinolytic fragments observed in this study. Although no direct conclusions can be made about the involvement of KLK7 in the production of this low-molecular weight gelatinolytic activity, the ability of KLK7 to produce similar gelatinolytic activities from MMP-9 *in vitro* warrants further investigation into this potential link.

In mixtures of gelatinases, the presence and activity MMP-9 and MMP-2 is often based solely on the size of the proteolytic products observed by gelatin zymography, with higher molecular weight products assigned as MMP-9 and lower molecular weight products designated as MMP-2. In view of the low-molecular weight proteolytically active MMP-9 produced by KLK7, identification of these gelatinases should likely include additional methodologies.

To determine whether the activation of proMMP-9 by KLK7 was specific to this gelatinase, we also tested the ability of KLK7 to activate proMMP-2. In contrast to the vigorous activation of MMP-9, no activation of proMMP-2 was observed upon incubation with KLK7 under similar reaction conditions. These results clearly show KLK7-dependent activation is specific for MMP-9, which is similar to the cleavage profiles observed for other proteolytic MMP activators.

MDA-MMP-9 cells have been used previously in studying activators of MMP-9 [11]. The level of proMMP-9 is much higher than TIMP-1 in these cells and they do not have detectable levels of MMP-2 [11] or *KLK7*[38]; thus, this cell line provides a good cell-based system to confirm the activation of proMMP-9 by KLK7. When active KLK7 was incubated with conditioned medium from these cells, a truncated, active fragment was produced similar to that observed using purified components. Interestingly, a second novel gelatinolytic band was also detected. A similar pattern of active MMP-9 products was also observed when purified proMMP-9 was incubated with KLK7 in SFM. The rapid activation of MMP-9 by KLK7 in these experimental settings suggests that KLK7 may play an important role in the activation of MMP-9 in tissues, where both MMP-9 and KLK7 have been reported to be overexpressed, such as pancreatic cancer.

To identify cellular factors that may regulate KLK7-activation of MMP-9, we employed MDA-MB-231 cells, the parental cell line used to generate the MMP-9-expressing cells. Incubation of proMMP-9 and KLK7 in MDA-MB-231 conditioned medium failed to produce the truncated active MMP-9 band and generated only very low levels of the second truncated fragment, observed with MDA-MMP-9 conditioned medium, suggesting the presence of an inhibitory factor. Since TIMP-1 is known to influence both the activation and activity of MMP-9, the MDA-MB-231 conditioned medium was immunodepleted specifically for TIMP-1 and KLK7-dependent MMP-9 activation was restored. The interaction of TIMP-1 with MMP-9 may prevent its dimerization and activation. Thus, though KLK7 is a novel activator of proMMP-9, the activation is attenuated by TIMP-1 similar to other proteases, suggesting that the balance between proMMP-9 and TIMP-1 cannot be circumvented by KLK7.

Interestingly, the possible link between activation of MMP-9 and a chymotryptic-like protease has been previously reported. In human skin, TNF-α-induced proteolytic activation of MMP-9 was found to be mediated by a chymotryptic-like serine protease and controlled by TIMP-1 [67]. Since KLK7 is a chymotrypsin-like protease predominantly expressed in human skin, it presents an intriguing possibility that KLK7 is responsible for the MMP-9 activation observed in this study.

We further used plasmin-activated KLK7 for testing KLK7-mediated MMP-9 activation to ensure that EDTA included to inhibit thermolysin did not alter the conformation of proMMP-9, thereby exposing sites that are usually not accessible for proteolytic activation of MMP-9 under physiological conditions. Additionally, plasmin activation of KLK7 clearly points out to the possibility of a novel proteolytic activation cascade (plasmin → KLK7 → MMP-9) in pathological states where all three proteases are expressed.

Though a clear role for the novel truncated MMP-9 we have observed is not known, it is possible that in neoplastic conditions these fragments could have possible pro-tumorigenic effects similar to those ascribed to the 83-kDa form of MMP-9 produced through the actions of other proteases. Most importantly this catalytically active fragment also lacks the C-terminal hemopexin domain, which is thought to mediate protein-protein interactions and contribute to substrate specificity; thus, this truncated MMP may engage additional substrates not targeted by full-length MMP-9.

In conclusion, the results of this study clearly indicate that unique low-molecular weight active MMP-9 fragments can be produced through the proteolytic action of KLK7 and suggests a novel role of KLK7 during its aberrant expression in human cancers, supporting the notion that it may play a significant part in tumor development and progression. Our present study highlights the need for understanding further the role of other protease families, like KLKs, in activating MMPs and the importance of taking into consideration such proteolytically active fragments while designing new therapies.

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